The Roles of Smad2 and Smad3 in Mouse Skin Development

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Abstract

The TGF-β cell signaling pathway functions in organism development, wound healing, skin function, hair growth, and tumorigenesis. Smad2 and Smad3 are proteins that specifically mediate TGF-β signaling. Previously in Dr. Weinstein's lab, a graduate student Mark Hester deleted Smad4, a mediator of TGF-β signaling downstream of Smad2 and Smad3, in skin fibroblasts. He observed defects in skin development and suggested that TGF-β signaling in fibroblasts was critical for skin development. Since Smad4 mediates BMP signaling as well as TGF-β, this project intended to determine the roles of Smad2 and Smad3 in skin development and tumorigenesis.

To demonstrate that defects in skin development of Smad4 mutants are due to TGF-β signaling, Smad2 and 3, which mediate TGF-β signaling through Smad4, were deleted in murine skin. Smad2 is deleted by crossing a Smad2 conditional allele with Cre transgenic mouse lines that express the protein either in fibroblasts or in the epithelium. Smad3 is introduced through a null allele. We hypothesized that if the skin defects in Smad4 mutant animals were due to defects in TGF-β signaling, and not because of defects in the BMP pathway also mediated by Smad4, then Smad2/Smad3 mutant mice would have similar skin defects as Smad4 mutant animals.

The first set of experiments involved Smad2 epithelial null/Smad3 null mutant animals. The mice are severely runted, exhibit abnormal wrinkly, scaly skin, hair loss, and die on day 16-18 after birth. Interestingly, this phenotype is more severe than what is observed in Smad4 mutants. Histological examination of the skin revealed hyperkeratinosis and abnormal hair follicle positioning and cycling. A second set of experiments involved Smad3 heterozygous mice with the Smad2 gene knocked out only in fibroblasts. Smad3 heterozygous mice were used because Smad3 null, Smad2 fibroblast-null animals die before birth. These mice initially appear normal;

however, they develop skin and oral cancerous lesions as adults. This finding is in agreement with others who have observed skin and oral lesions in Smad4 mutant animals.

Comparison of skin and tumor histology using H&E staining revealed similarities in the phenotypes of Smad4 and Smad2/Smad3 mutants. TUNEL staining, a technique that marks apoptotic cells revealed increased levels of apoptosis in the skin of mutants. Ki67 imminohistochemistry, a marker for dividing cells, revealed cell proliferation abnormalities in the mutants. Masson's trichrome staining detected decreases in skin collagen fibers in epithelial mutants. Skin lesion progression was visualized microscopically with H&E staining. Lastly, RT-PCR was performed on skin samples and tumors of Smad3 heterozygous, Smad2 fibroblast-null mutant mice to assess the level of TGF-β transcription. Expression of several TGF-β target genes was shown to be up-regulated in tumors of *FSP:Cre; Smad2fl/fl;Smad3+/-* mice including: fibronectin, MMP-2, MMP-9, Snail, and Smad7.

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Introduction

TGF-β Pathway

The transforming growth factor-beta (TGF-β) is a ubiquitous superfamily of ligands consisting of a protein set that regulates a variety of processes during prenatal development, including cell differentiation, development, angiogenesis, cell proliferation, and apoptosis. In adult organisms TGF-β regulates immune responses and homeostasis, promotes wound healing, and either suppresses or promotes tumorigenesis.

The TGF-β signaling pathway begins when an extracellular ligand binds to a transmembrane type II receptor at the cell surface [Figure 1], which activates the serine/threonine kinase domain of the receptor. The activated receptor then recruits a complimentary transmembrane Type I receptor and phosphorylates its glycine/serine domain. The phosphorylation activates Type I receptor's serine/threonine kinase, which then recognizes and phosphorylates a Serine Serine X Serine (SSXS) motif on the Mad homology 2 (MH2) domain of receptor-regulated Smad proteins (rSmads), Smad2 and Smad3. After phosphorylation, either one of the rSmads can form a complex with the co-Smad, Smad4 [2, 3]. The r-Smad-co-Smad complex then migrates to the nucleus where it activates or represses target gene transcription [1]. The complex will bind either with transcriptional co-activators (p300) and promote transcription, or with transcriptional co-repressors (TGIF) and repress transcription [2]. It should be noted that Smad4 can also regulate signaling from other pathways, such as bone morphogenic protein (BMP) and activin pathways through a separate group of rSmads, including Smad1,-5, and –8.

Interest in Smad proteins and TGF-β signaling in general has skyrocketed following several reports implicating a role of various Smads in tumorigenesis. Abnormalities in all Smads

of the TGF-β/activin pathway have been reported in various tumors. For example, the common Smad, Smad4, has been shown to be mutated in 50% of pancreatic tumor cases [4, 5]. Smad2 is under expressed in head and neck carcinomas, while some lung and colorectal cancers have been related to a null Smad2 mutation ([6], [7]). The loss of Smad3 is linked to increasing susceptibility to human gastric cancer [8], while Smad3 null mice develop metastatic colorectal cancer [9].

The role of TGF-β in skin development and healing continues to be a topic of active research. Studies have shown that TGF-β is involved in the activation of fibroblasts, which are the main collagen-producing cells and are therefore essential to skin development and maintenance, particularly the dermis layer [1]. Research with wounds and burns has consistently shown that TGF-β is capable of speeding up the healing process not only if applied topically after injury, but also if administered internally before the injury occurs [3].

The role of Smad4, the mediator of TGF-β pathway, has been investigated by Dr. Weinstein's graduate student Mark Hester. As a part of his doctorate dissertation, he studied mutant mice in which Smad4 was deleted in skin fibroblasts. The animals had wrinkly, scaly skin, complete loss of hair by day 40, and most of them died by day 50. Upon histological examination of the skin, Mark found that the mutants had multiple dermal cysts, hyperkeratinosis, and enlarged, disoriented hair follicles that did not properly go through hair follicle development cycle. Immunohistochemistry results showed both increased proliferation and decreased apoptosis of keratinocytes around hair follicles. Mark concluded that Smad4 is essential for proper hair follicle functioning. Specifically, he implicated Smad4 in follicles' entering of the catagen stage (a stage in which cell death normally takes place), as well as cell

proliferation control. Mark also hypothesized, but never tested, that Smad2 and Smad3 together might function in the patterning of the endoderm layer of the skin.

The purpose of this project was to specifically investigate the roles of Smad2 and Smad3 in mouse skin development and to compare observed phenotypes of Smad2 and Smad3 conditional mutants to that of Smad4. Mice that are Smad2 null or Smad2 heterozygous in combination with either Smad3 null or heterozygous are not viable. When only one of the Smads is lacking (either $Smad2^{+/-}$ or $Smad3^{-/-}$ genotype), the animals survive. Therefore, an approach involving a combination of Smad2 with Smad3 mutations should be used in order to characterize the phenotypes and infer the function of Smad2 and Smad3.

Another option is to create conditional knockouts with Cre-LoxP system. Cre recombinase of the P1 bacteriophage is a member of the integrase family of site-specific recombinases. Cre recombinase catalyzes recombination between two recognition sites called loxP sites. The use of Cre-LoxP system allows for creation of conditional knockouts. In this project, FSP and MMTV conditional knockouts were introduced into Smad2. FSP-1 is fibroblast-specific protein-1, and FSP-1:Cre is a fibroblast-specific knockout, which deletes Smad2 in fibroblasts of transgenic animals. In his unpublished dissertational work, Mark Hester showed successful deletion of Smad4 with FSP-1:Cre in mice. Specifically, mice lacking Smad4 in fibroblasts were obtained by crossing mice carrying a Smad4-null conditional allele to transgenic animals carrying the Cre recombinase transgene under the control of the FSP-1 promoter. MMTV is a mouse mammary tumor virus. MMTV:Cre knockouts delete Smad2 in mammary tissue and skin. In order to create *FSP:Cre;* $Smad2^{fl/fl}$*;*$Smad3^{+/-}$ and *MMTV:Cre;* $Smad2^{fl/fl}$*;* $Smad3^{-/-}$ genotypes used in this project, Smad2 is deleted by crossing a Smad2 conditional allele

with Cre transgenic mouse lines under the control of either FSP-1 or MMTV promoter. Smad3 is introduced through a null allele.

Skin

Mammalian skin consists of 3 major layers: epidermis (top layer), dermis (middle layer), and hypodermis (deepest layer) [Figure 2]. The epidermis is the outer layer of skin with variable thickness between species. It contains 5 layers: strata basale, spinosum, granulosum, lucidum, and corneum. Stratum basale, the deepest layer, has column-shaped cells, which divide, move up to higher layers, and flatten out. Stratum corneum, the top layer, is made of dead cells (keratinocytes) that shed periodically. The dermis also varies in thickness. It consists of collagen, elastic extracellular matrix, and reticular fibers, and is divided into reticular and papillary layers. Hair follicles, sebaceous glands, sweat glands, apocrine glands, and blood vessels. Beneath the dermis lies the hypodermis, which is not considered to be part of skin, but rather subcutaneous tissue. The hypodermis consists mainly of loose connective tissue and adipocytes [30].

Results

General condition of mutant animals

Comparison between Smad4 and Smad2/Smad3 mutant animals was made on various levels. On a macroscopic scale, adult mice were compared in terms of size, skin, hair, and tumor presence [Figure 3]. *FSP:Cre; Smad4$^{fl/fl}$* mice are significantly smaller in size compared to wild type. By day 65, they exhibit wrinkly, scaly skin and loss of hair. *MMTV:Cre; Smad4$^{fl/fl}$* mutant mice are not as runted as *FSP:Cre; Smad4$^{fl/fl}$,* but also exhibit wrinkly, scaly skin and loss of hair. *MMTV:Cre; Smad2$^{fl/fl}$; Smad3$^{-/-}$* phenotype resembles both of the Smad4 mutant phenotypes. Again, mutant animals are runted, with skin abnormalities and hair loss. In contrast, *FSP:Cre; Smad2$^{fl/fl}$;Smad3$^{+/-}$* mutants do not lose hair but develop oral tumors and skin lesions as adults.

Comparison of skin histology

A second level of comparison involved H&E staining of skin sections of *FSP:Cre; Smad2$^{fl/fl}$;Smad3$^{+/-}$, MMTV:Cre; Smad2$^{fl/fl}$; Smad3$^{-/-}$, MMTV:Cre; Smad4$^{fl/fl}$,* and *FSP:Cre; Smad4$^{fl/fl}$* mutants. Stained sections of mutant and wild-type mice were photographed and compared.

In wild-type skin, epidermis, dermis, and hypodermis are normally-thick [Figure 4-a]. There is a thin keratin layer on top of the epidermis, which corresponds to proper layering. Hair follicles are originating in the hypodermis and extending into the dermis - their proper orientation. *FSP:Cre; Smad4$^{fl/fl}$* mutant exhibits keratin build up in the epidermis/dermis layer. Hair follicles seem disoriented: they are not oriented in the hypodermis as in the wild-type and their positioning is quite random [Figure 4-b]. *MMTV:Cre; Smad4$^{fl/fl}$* skin shows epidermal

hyperplasia, as well as overall disruption of epidermal layer structure [Figure 4-d]. *FSP:Cre; Smad2$^{fl/fl}$;Smad3$^{+/-}$* skin exhibits major epidermal hyperplasia and disoriented hair follicles in the dermis layer [Figure 4-c]. *MMTV:Cre; Smad2$^{fl/fl}$; Smad3$^{-/-}$* skin shows epidermal hyperplasia and hyperkeratinosis [Figure 4-e], both features found in *FSP:Cre; Smad4$^{fl/fl}$;* mutant.

Based on the observations of skin histology, both Smad4 mutants exhibit abnormalities comparable to those observed in Smad2/Smad3 mutant animals. Epidermal hyperplasia is common between *MMTV:Cre; Smad4$^{fl/fl}$* and both Smad2/Smad 3 mutants. Hyperkeratinosis is common between *FSP:Cre; Smad4$^{fl/fl}$* and *MMTV:Cre; Smad2$^{fl/fl}$; Smad3$^{-/-}$* mutants. Hair follicle disorientation is common between both fibroblast mutants.

In addition to H&E, Masson's Trichrome staining was performed in order to compare collagen levels in *MMTV:Cre; Smad2$^{fl/fl}$; Smad3$^{-/-}$* mutant skin to wild-type skin [Figure 5]. The procedure revealed significant decrease in collagen levels in mutant animals. The decrease explains the wrinkly and non-elastic nature of the mutant skin.

Apoptosis and proliferation level assessment

Since TGF-β is known to play a role in apoptosis and cell proliferation, levels of both were assessed and compared in *FSP:Cre; Smad4$^{fl/fl}$, FSP:Cre; Smad2$^{fl/fl}$;Smad3$^{+/-}$,* and *MMTV:Cre; Smad2$^{fl/fl}$; Smad3$^{-/-}$* mutant skin. Levels of apoptosis were assessed using TUNEL staining procedure, which labels DNA strand breaks that occur during apoptosis. The results revealed increased apoptosis of cells in the skin, especially around hair follicles, in *FSP:Cre; Smad4$^{fl/fl}$, FSP:Cre; Smad2$^{fl/fl}$;Smad3$^{+/-}$,* and *MMTV:Cre; Smad2$^{fl/fl}$; Smad3$^{-/-}$* mutants. Apoptosis levels in wild-type skin were minimal [Figure 6]. *MMTV:Cre*; *Smad2$^{fl/fl}$;Smad3$^{-/-}$* mutant seemed to show the highest levels of apoptosis. Even though increased apoptosis was shown in the

samples used, it should be noted that skin exhibits different levels of apoptosis depending on the stage of the hair follicle cycle. Therefore, in order to assess the levels of apoptosis between mutants with certainty, larger sample with a control for timing is needed.

Ki67 imminohistochemistry was done to assess the levels of cell proliferation. Although in some cases there appeared to be a difference in proliferation between the mutants, there were not enough samples to confirm the results. It is possible that the animals were sampled at different stages of the hair follicle cycle, and therefore the results captured typical proliferation levels of different stages.

Oral tumors, skin lesions, and lung metastases

Both Smad4 and Smad2/3 mutant types exhibit oral tumors. In *MMTV:Cre; Smad2$^{fl/fl}$; Smad3$^{-/-}$* and *FSP:Cre; Smad2$^{fl/fl}$;Smad3$^{+/-}$* mutants used in this project, oral tumors metastasized to lungs. Anywhere from 1 to a dozen lung metastases were found at a time. Lungs are a typical metastasis place in mice, just like bone is typical for humans. Usually one or a couple skin lesions were observed on both Smad2/Smad3 mutants. The lesions are characteristic of squamous cell carcinomas: they resemble an open wound with occasional bleeding.

Oral Tumor and Skin Lesion Histology

The next step involved comparing oral tumors and skin lesions on a microscopic scale. First, histology of oral tumors was compared for *FSP:Cre; Smad4$^{fl/fl}$*, *FSP:Cre; Smad2$^{fl/fl}$;Smad3$^{+/-}$,* and *MMTV:Cre; Smad2$^{fl/fl}$; Smad3$^{-/-}$* mutants using H&E staining [Figure 9]. Tumors from Smad2 and Smad3 mutant animals exhibit common features. The tumor from *FSP:Cre; Smad4$^{fl/fl}$* mutant resembles *MMTV:Cre; Smad2$^{fl/fl}$; Smad3$^{-/-}$*. Such results might imply a

similar nature of tumors in the three mutants; however, more samples would be needed to allow for deeper comparison. It should be noted that *FSP:Cre; Smad4$^{fl/fl}$* animal tumors develop much later than the tumors in Smad2/Smad3 mutants. Therefore, their histology would be expected to differ.

Next, progression of skin lesions from normal skin to carcinomas was illustrated with H&E staining of three different skin sections from *FSP:Cre; Smad2$^{fl/fl}$;Smad3$^{+/-}$* and *MMTV:Cre; Smad2$^{fl/fl}$; Smad3$^{-/-}$* mutants [Figure 10]. The first section is normal skin that was taken from a mutant animal with progressed carcinoma, at least 1 inch from the actual lesion. Even though the skin appears normal on the surface, epidermal hyperplasia is already revealed with histology. Small nodules are also beginning to build up. The second section was taken closer to the border between normal-looking skin and the lesion. Finally, the third section was taken from the lesion itself. Overall, *FSP:Cre; Smad2$^{fl/fl}$;Smad3$^{+/-}$* mutant exhibits more dramatic pathology: epidermal hyperplasia is more severe and there is complete disruption of the epidermis layer in the lesion itself. Epidermis disruption is characteristic of carcinoma. Though not as severe, *MMTV:Cre; Smad2$^{fl/fl}$; Smad3$^{-/-}$* mutant also exhibits major skin abnormalities leading to lesions. Slight epidermal hyperplasia is observed. The size of the nodules is also progressively increasing closer to the lesion.

TGF-β target gene transcription

Finally, levels of transcription of TGF-β target genes in tumors versus skin of *FSP:Cre; Smad2$^{fl/fl}$;Smad3$^{+/-}$* mutants were assessed with RT-PCR [Figure 11]. L34 is a transcript of a ribosomal protein and served as a control to show high expression in both tumors and skin. Snail is a gene involved in epithelial-mesenchymal transition and is expressed in invasive tumors. The gene was up-regulated in tumors, while its expression was absent in the skin. Mmp-2 and Mmp-9 are matrix metallo-proteinases. They degrade extracellular matrix around tumors and promote tumor invasion. Mmp-2 showed slight expression in the skin, but the expression in tumors was much higher. Mmp-9 showed elevated expression in tumors only. Smad7 is an inhibitor of TGF-β and is up-regulated in response to TGF-β signaling. Its expression was also up-regulated in tumors and absent in the skin. Fibronectin is a component of extracellular matrix, and was expressed in both tumors and skin, with slightly more expression in tumors.

Discussion

Macro- and microscopic examination of Smad4 and Smad2/Smad3 mutants reveals similarities in phenotypes

As mentioned earlier, the major goal of this project was to compare the functional roles of Smad4 versus Smad2 and Smad3 in three classes of mutants: *FSP:Cre; Smad4$^{fl/fl}$, MMTV:Cre; Smad2$^{fl/fl}$; Smad3$^{-/+,}$ and FSP:Cre; Smad2$^{fl/fl}$;Smad3$^{+/-}$*. The roles of Smad2 and Smad3 in mouse skin development were assessed by observing the phenotypes of Smad2/Smad3 mutant alleles. Although not as insightful, similarities in general appearance of the three mutant animals started this project on track to more levels of comparison. Deletion of either Smad4, or Smad2 and Smad3 in conjunction resulted in wrinkly, scaly skin, severe growth retardation, and hair loss.

Skin and tumor histochemistry results revealed that, although not identical, the phenotypes of *FSP:Cre; Smad4$^{fl/fl}$, MMTV:Cre; Smad2$^{fl/fl}$; Smad3$^{-/+,}$ and FSP:Cre; Smad2$^{fl/fl}$;Smad3$^{+/-}$* show multiple similarities. Hyperkeratinosis, epidermal hyperplasia, dermal cysts, and hair follicle disorientation are the main defining features of the skin of all three mutants. Tumor histology analysis, where tumors from *FSP:Cre; Smad4$^{fl/fl}$, MMTV:Cre; Smad2$^{fl/fl}$; Smad3$^{-/+,}$ and FSP:Cre; Smad2$^{fl/fl}$;Smad3$^{+/-}$* mutant animals were compared, revealed major similarities between the two Smad2/Smad3 mutants. Smad4 mutant histology was similar to *MMTV:Cre; Smad2$^{fl/fl}$; Smad3$^{-/+,}$* in some aspects. However, more samples have to be compared to allow for a more complete description. As previously stated, Smad4 is a Smad common to all TGF-β superfamily pathways, while Smad2 and Smad3 exclusively mediate TGF-β and activin. Based on the phenotype similarities we saw in the skin, we concluded that the defects that Mark Hester observed in his *FSP:Cre;Smad4$^{fl/fl}$* mutants can be attributed to defects in TGF-β signaling, and not BMP signaling which is also mediated by Smad4.

Oral Tumors

Oral tumors observed in mutant animals are unique because they are the first case of oral tumors from a genetic knockout. Previous research has shown that TGF-β over-expression can aid in pre-existing tumor regression [28], but it was never shown to be the cause of oral tumors. Moreover, the oral tumors in this study were shown to metastasize to lungs, another unexpected finding.

An interesting result was up-regulation of TGF-β target genes in tumors compared to the skin, especially considering the fact that over-expression of TGF-β has been shown to cause tumor regresson. [28]. Even though TGF-β regulators were inhibited, TGF-β target genes were still

upregulated, which was quite unexpected. Further research is needed to explain this result. For now, an unknown compensatory mechanism with a way to turn on TGF-β despite the r-Smad knockout was suggested.

Overall, the results presented in this paper show an independent effect of Smad2/Smad3 and Smad4. Smad2/Smad3 mutants exhibit a more severe phenotype in some aspects, such as early oral tumor growth and skin abnormalities. Moreover, *FSP:Cre; Smad4*$^{fl/fl}$ mutants are viable, while *FSP:Cre;Smad2fl/fl; Smad3-/-* are embryonic lethal. This suggests that Smad2/Smad3 mutant phenotype is a lot more severe, and that there is a possibility of Smad2/Smad3 independent participation. Perhaps this finding could challenge the existing knowledge of the pathway – that Smad2 and Smad3 are upstream and do less in TGF-β than Smad4. It should be noted that *FSP:Cre; Smad4*$^{fl/fl}$ mutant used for comparison in this study is conditional, while *FSP:Cre;Smad2fl/fl; Smad3-/-* mutant is constitutive, which might partially explain why Smad2/Smad3 phenotype is more severe [33]. However, a conclusion of their independent action can still be made, and this project was the first to show such action in both tumors and skin.

Future Direction

Just because our conditional Smad2/Smad3 mutants produced a phenotype similar to the one produced by Smad4 conditional mutant, we cannot with certainty conclude that the defects observed in *FSP:Cre; Smad4*$^{fl/fl}$; are due solely to TGF-β signaling defects. The pathway could act synergistically with other TGF-β pathways and their components. Therefore, a suggestion for future research would be to create the same conditional knock-outs for Smads that specifically mediate the BMP pathway, such as Smad1, Smad5, and Smad8. Such knockouts are feasible, but have not been done. It would be interesting to see how mutant phenotypes of Smad1, Smad5, and Smad8 individually or in combination would compare to both Smad4 and Smad2/Smad3 conditional mutants.

Materials and Methods

Genotyping of mice: Agarose gel electrophoresis was used to analyze all samples after PCR amplification.

Toe DNA: DNA was extracted from toes of mice using a lysis/buffer system.

PCR: Smad2 genotyping was done with primers AA and left. Smad3 genotyping was done with primers 5 and 7. Smad4 genotyping was done with primers CoA-1 and CoB-1. Cre-1 genotyping was done with primers 1 and 3 (Table 2). Reactions are run with 35 cycles with an annealing temperature of 55°C.

RT-PCR: RNA was isolated from tissue flash frozen on dry ice using TRIzol reagent (Invitrogen) according to manufacturer's protocol. cDNA was prepared using a SuperScript III First-Strand Synthesis System (Invitrogen). Semi-quantitative reverse transcriptase (RT) PCR was used to assess expression of TGF-ß target genes following induction by TGF-ß1 treatment**.** Performed with primers listed in table 3.

Hematoxylin and Eossin (H&E) stain: Done in the lab following a regular staining protocol.

Masson's Trichrome: Done at the pathology core facility, The Ohio State University College of Veterinary Medicine

Ki67 Imminohistochemistry: Assesses cell proliferation by the detection of Ki67 antigen. The polyclonal antibody (NCL-Ki67p) labels Ki67 antigen during late G1, S, G2 and M stages of cell cycle. Reagents: Ki67 anti-body, 1:100 dilution (Santa Cruz, sc 7846, #K3004 goat polyclonal IgG), Vectastain ABC kit (Vector labs, PK-6105), DAB kit (Vector labs SK-4100). Performed following manufacture's protocol.

TUNEL: Detects and quantifies apoptosis based on labeling of DNA strand breaks. Cleavage of DNA during apoptosis is identified by labeling free 3'-OH terminal with modified nucleotides in an enzymatic reaction. Reagents: Proteinase K, TdT, FITC-Avidin D. Performed following manufacture's protocol.

Figures and Tables

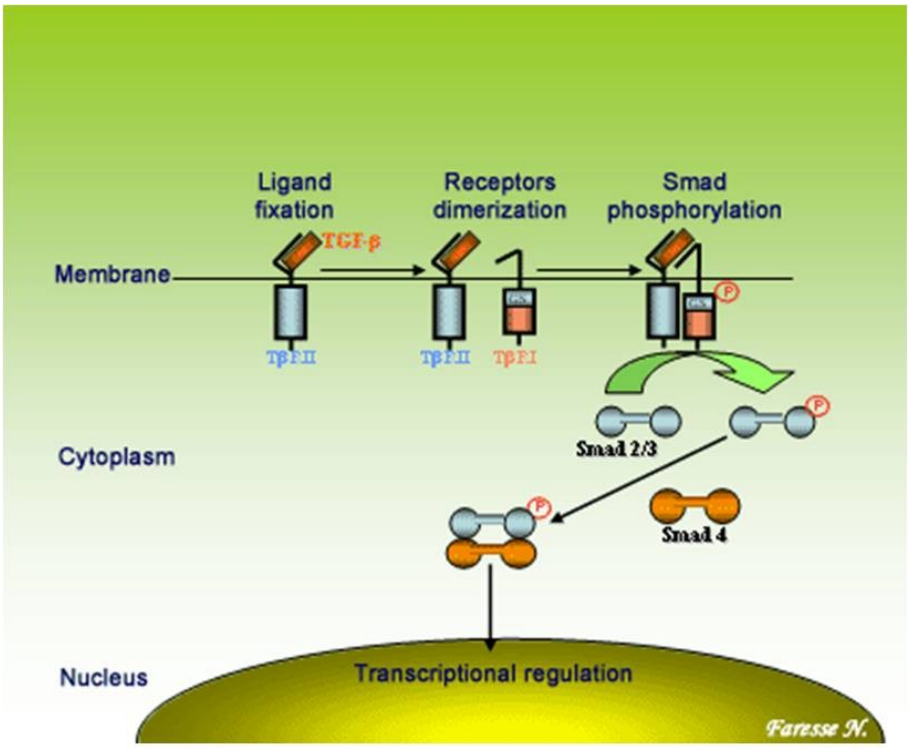

Figure 1. TGF-β signaling pathway

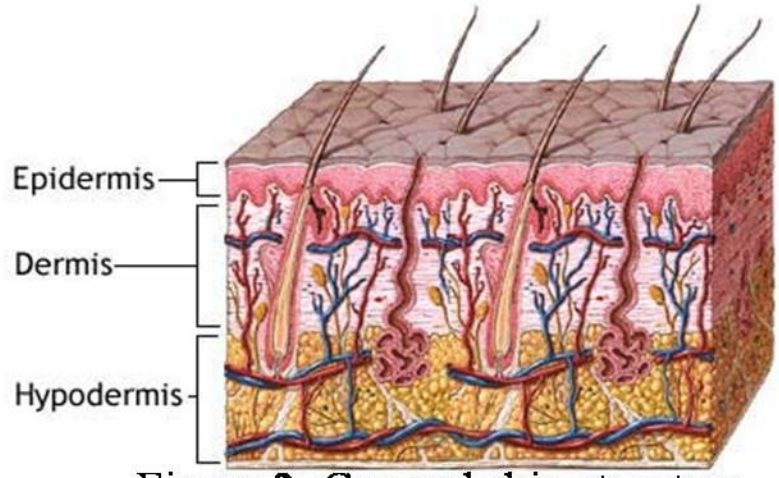

Figure 2. General skin structure

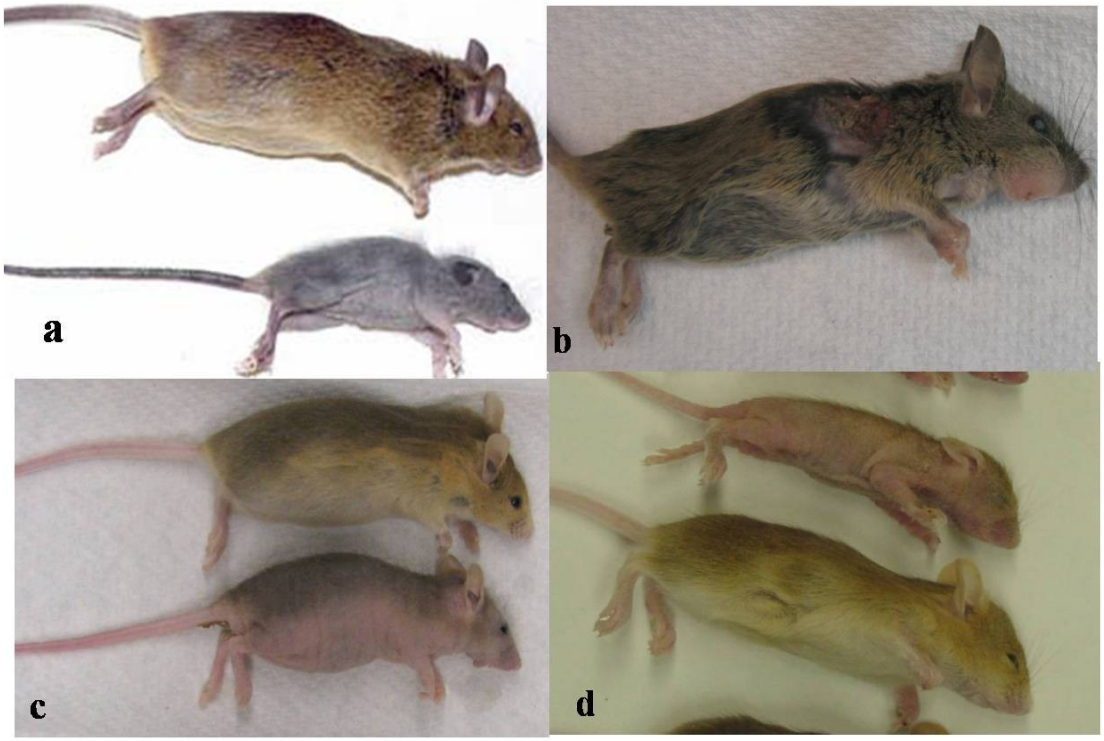

Figure 3. Phenotypes of mutant animals.**a.** *FSP:Cre; Smad4$^{fl/fl}$;* **b.** *FSP:Cre; Smad2$^{fl/fl}$;Smad3$^{+/-}$* **c.** *MMTV:Cre; Smad4$^{fl/fl}$* **d.** *MMTV:Cre; Smad2$^{fl/fl}$; Smad3$^{-/-}$*

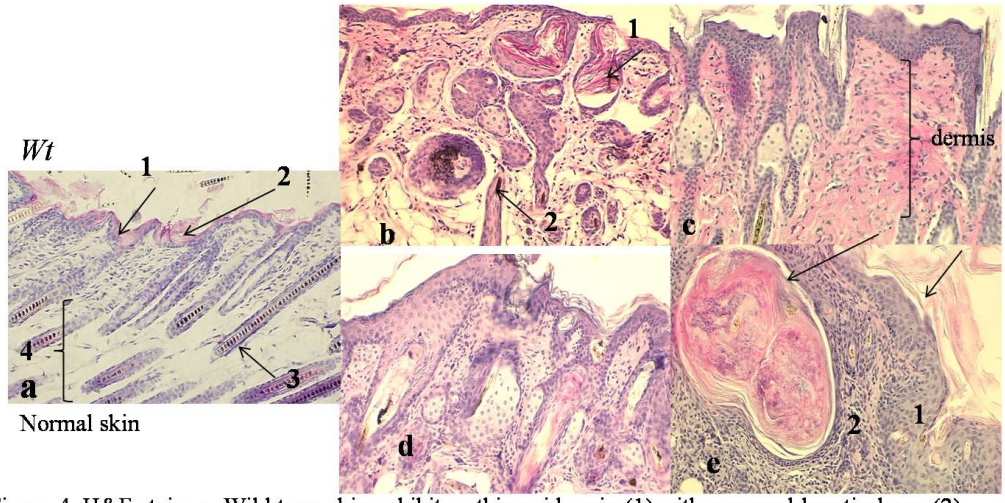

Figure 4. H&E stain. **a.** Wild-type skin exhibits a thin epidermis (1) with a normal keratin layer (2). Hair follicles (3) are properly oriented in the hypodermis (4). **b.** *FSP:Cre; Smad4$^{fl/fl}$* mutant skin shows hyperkeratinosis (1) and disoriented hair follicles (2) **c.** *FSP:Cre; Smad2$^{fl/fl}$;Smad3$^{+/-}$*mutant skin shows epidermal hyperplasia,and disoriented hair follicles **d.** *MMTV:Cre; Smad4$^{fl/fl}$* mutant skin with epidermal hyperplasia and displaced hair follicles (3) **e.** *MMTV:Cre; Smad2$^{fl/fl}$; Smad3$^{-/-}$*mutant skin exhibits epidermal hyperplasia (1) with hyperkeratinosis (2)

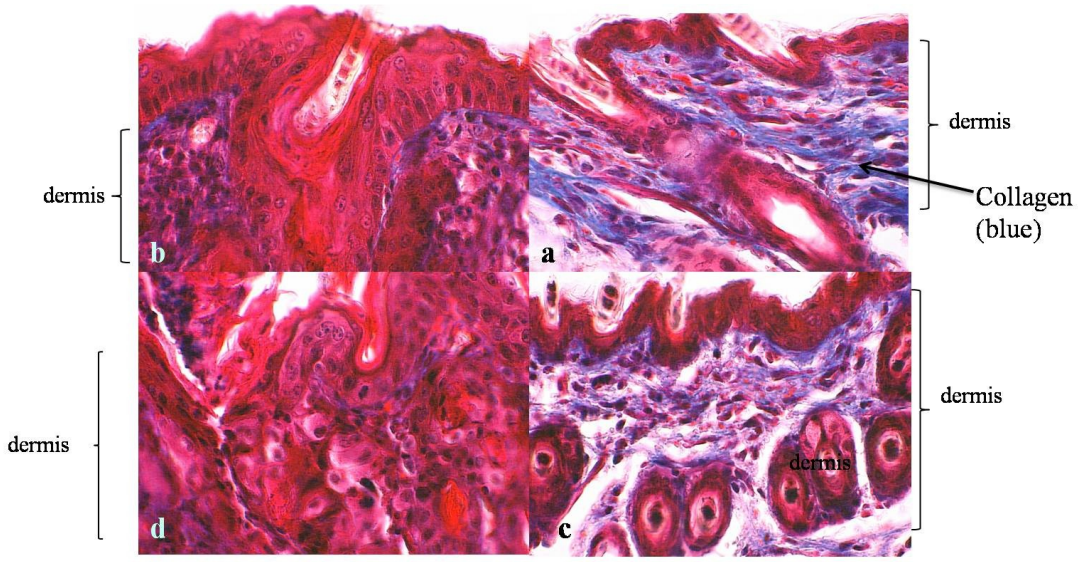

Figure 5. Masson's Trichrome. Decreased collagen (stained blue) in the dermis of *MMTV:Cre; Smad2$^{fl/fl}$; Smad3$^{-/-}$*
mutant skin (**b,d**) compared to wild-type (**a,c**)

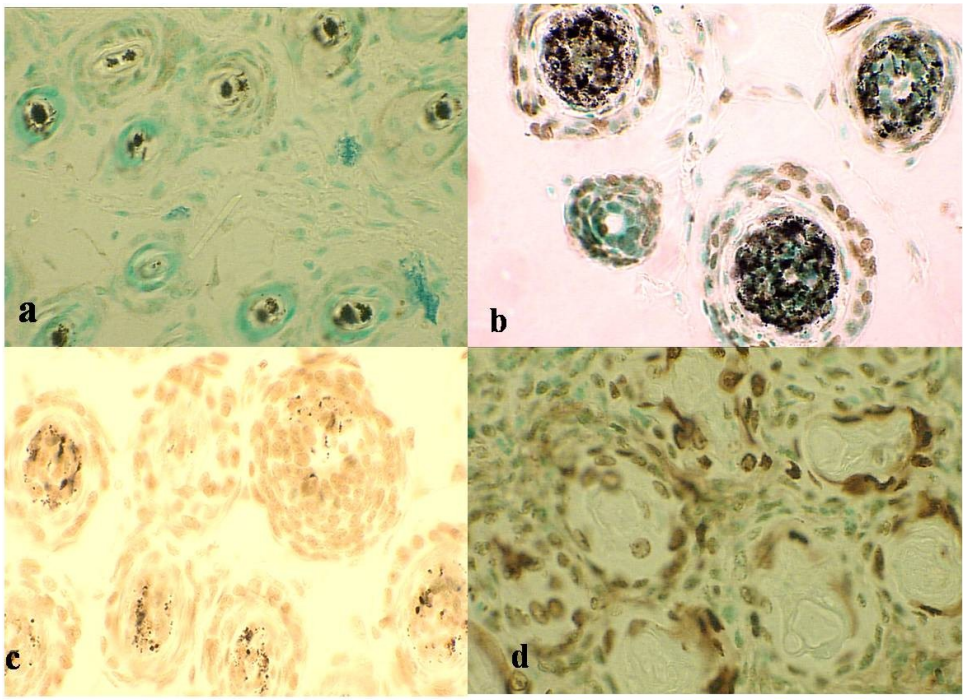

Figure 6. TUNEL staining. Increased apoptosis is observed around hair follicles in mutant animals: **b.** *FSP:Cre; Smad2$^{fl/fl}$;Smad3$^{+/-}$* **c.** *FSP:Cre; Smad4$^{fl/fl}$;* **d.** *MMTV:Cre; Smad2$^{fl/fl}$; Smad3$^{-/-}$* Normal levels observed in wild-type skin: **a.**

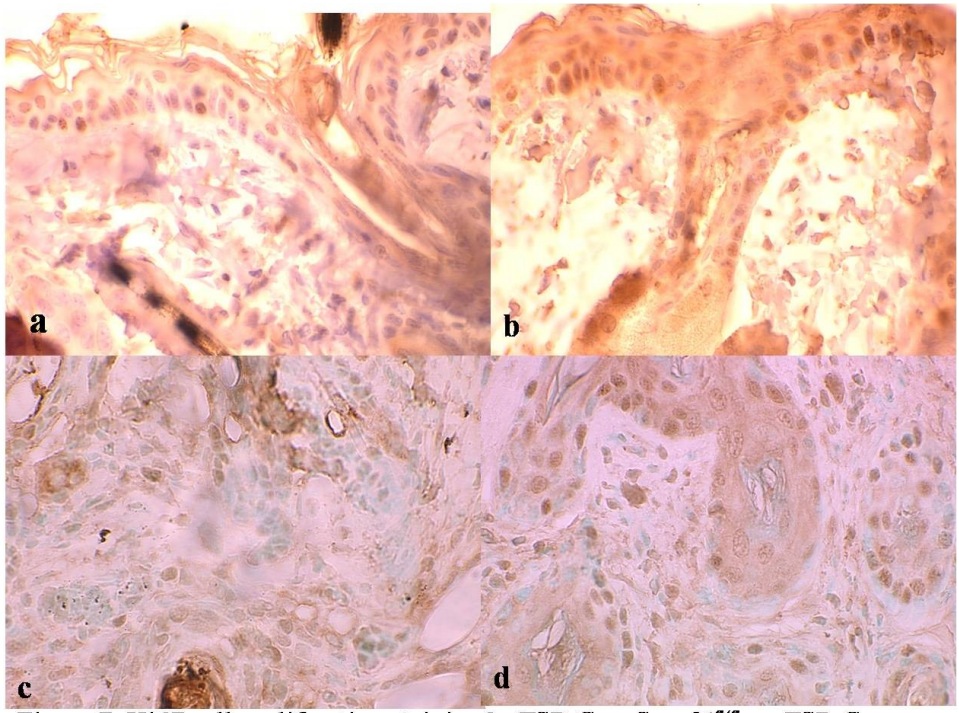

Figure 7. Ki67 cell proliferation staining **b.** *FSP:Cre; Smad4$^{fl/fl}$;* **c.** *FSP:Cre; Smad2$^{fl/fl}$;Smad3$^{+/-}$* **d.** *MMTV:Cre; Smad2$^{fl/fl}$; Smad3$^{-/-}$* Normal levels observed in wild-type skin: **a.** Wild-type

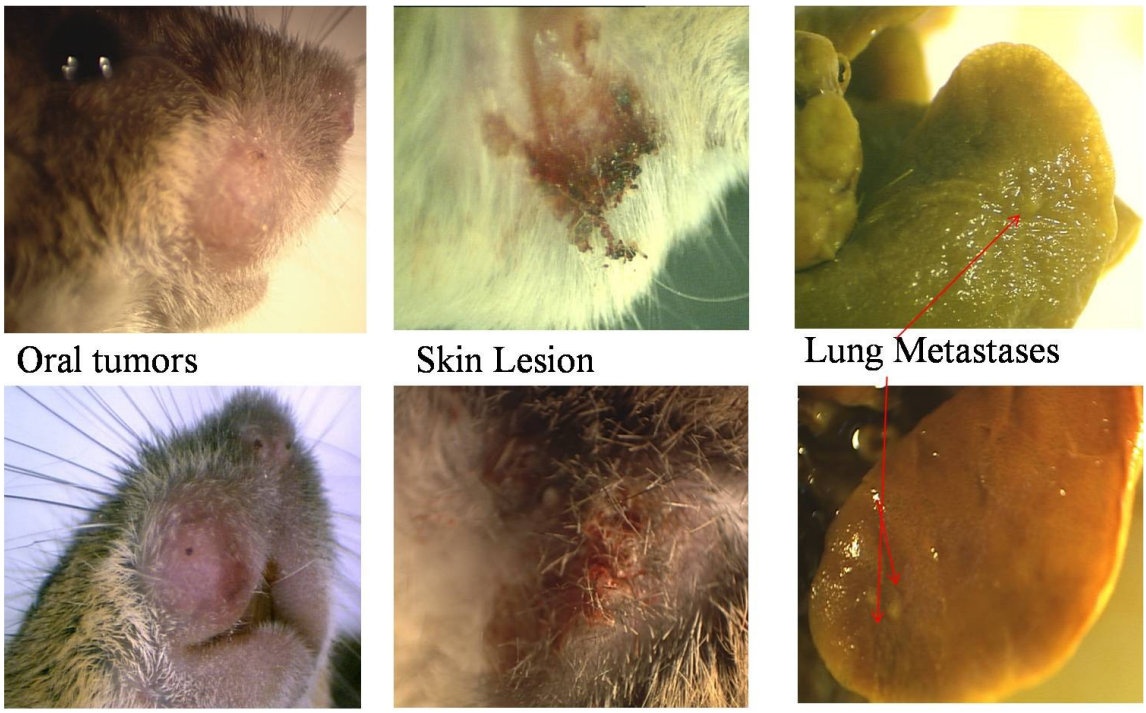

Figure 8. Oral tumors, skin lesions, and lung metastases of *FSP:Cre; Smad2$^{fl/fl}$;Smad3$^{+/-}$* (bottom row)and *MMTV:Cre; Smad2$^{fl/fl}$; Smad3$^{-/-}$* (top row) mutants

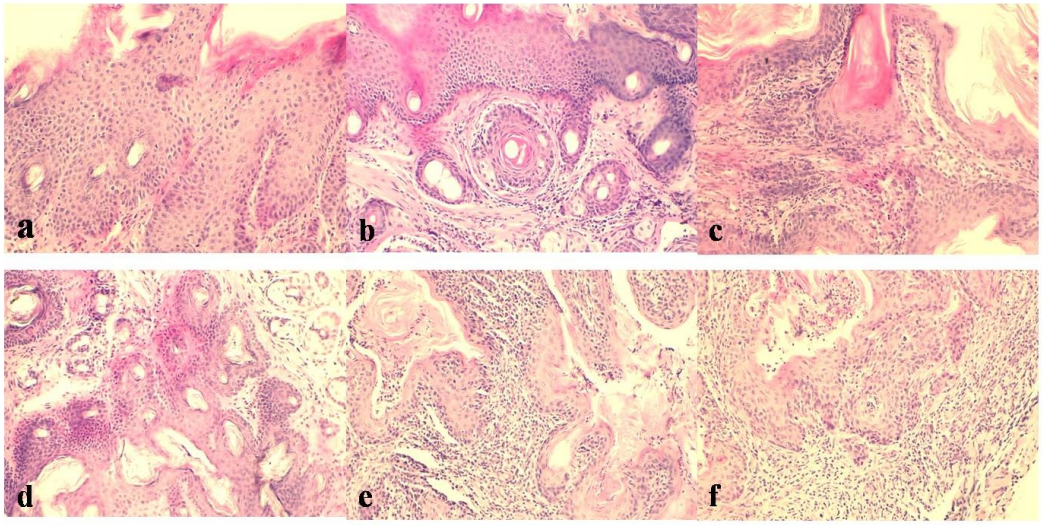

Figure 9. H&E stain. *FSP:Cre; Smad4$^{fl/fl}$* mutant (**c,f**) oral tumor histology resembles both *FSP:Cre; Smad2$^{fl/fl}$;Smad3$^{+/-}$* (**a,d**) and *MMTV:Cre; Smad2$^{fl/fl}$; Smad3$^{-/-}$* (**b,e**) mutants.

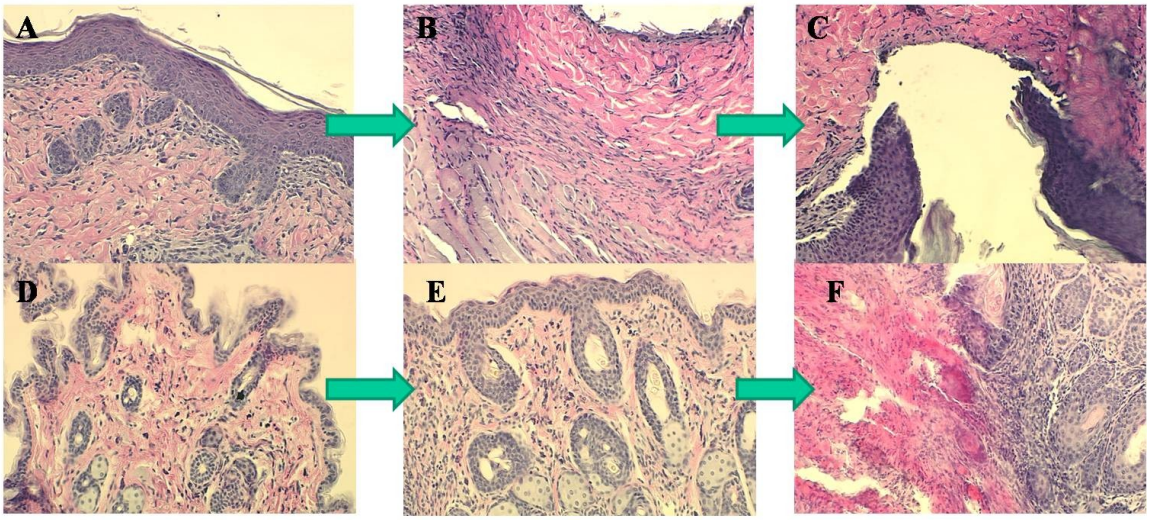

Figure 10. H&E stain of skin lesions. **A, D**: normal-looking skin from an animal with lesions; **B, E**: normal skin/lesion border: **C, F**: lesion **Top:** *FSP:Cre; Smad2$^{fl/fl}$;Smad3$^{+/-}$*
Bottom: *MMTV:Cre; Smad2$^{fl/fl}$; Smad3$^{-/-}$*

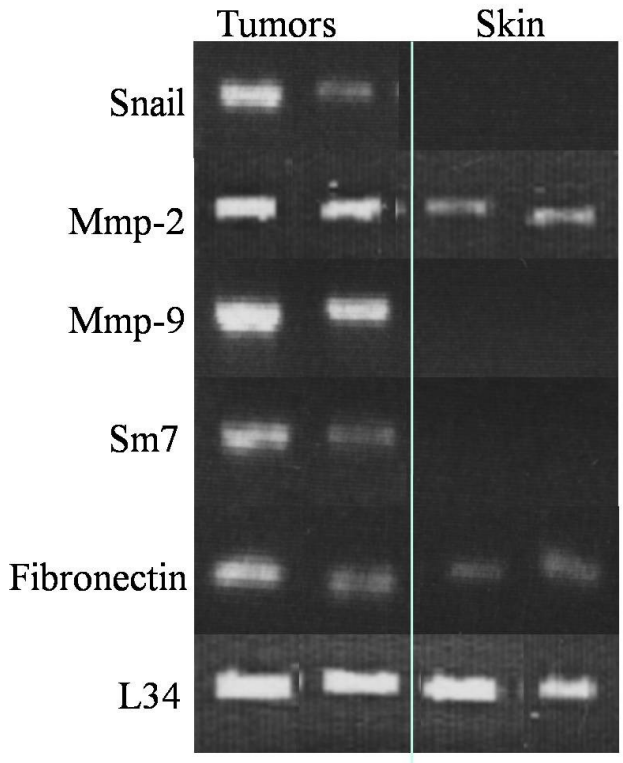

Figure 11. RT-PCR Results. Expression of TGF-ß target genes is up-regulated in tumors of *FSP:Cre; Smad2$^{fl/fl}$;Smad3$^{+/-}$* mice. Snail is a gene involved in epethelial-mesenchymal transition and is expressed in invasive tumors. Mmp-2 and mmp-9 are matrix metallo-proteinases. They degrade extracellular matrix around tumors and promote tumor invasion. Smad7 is an inhibitor of TGF-ß signaling and is up-regulated in response to TGF-ß signaling. Fibronectin is a component of extracellular matrix. L34 is a transcript of a ribosomal protein and serves as a control.

	Smad2 $^{+/+}$	Smad2 $^{+/-}$	*MMTV:Cre; Smad2$^{fl/fl}$*	*FSP:Cre; Smad2$^{fl/fl}$*
Smad$^{+/+}$	Wt	Wt	Wt	Wt
Smad3$^{+/-}$	Wt	Wt	Skin abnormalities	Skin abnormalities and oral tumors
Smad3$^{-/-}$	Wound healing phenotype	Embryonic lethal	Skin abnormalities and oral tumors	Embryonic lethal

Table 1. Corresponding viability phenotypes of Smad2/Smad3 combinations of alleles

Table 2. Primers used to genotype mice.

Primer	*Sequence 5'-3'*
CRE-1	CGTGTTTTGCACGTTCACCG
CRE-3	ATGCTTCTGTCCGTTTGCCG
Smad2-AA	GTCACTCCCTGAACCTGAAG
Smad2-Left	TACTTGGGGCAATCTTTTCG
Sm3-5	CAACTTCATTGCCATATGCCCT
Sm3-7	CCCGAACAGTTGGATTCACACA
Sm4CoA-1	AAGAGCCACAGGGTCA
Sm4 CoB-1	TTCCAGGAAAAACAGGGCTA

Gene	Forward sequence	Reverse sequence
PAI-1	GCCAGGGTTGCACTA AACAT	CTCGAGTATGACGTC GTGGA
Smad7	ATCTCAGGCATTCCTC GGAAG	CAGCCCTTCACAAAG CTGATC
MMP-2	CACATCCTTCACCTGGT GTG	GCACTCTGGAGCGAGGA TAC
MMP-9	GGAATGATCTAAGCCCA GTGCAT	GACCCGAAGCGGACATT GTCAT
Fibronectin	TGAGTGCTTCATGCCGTT AG	AAGTCTCAAACATCCCA CGG
L34	CCTTCTCTGGAACAACC TTCTCG	AAGATGATGAACACCGA CCTTAGC

Table 3: RT-PCR primer sequences used for TGF-β target gene transcription assessment

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